Expression vectors for enzyme restriction- and ligation-independent cloning for producing recombinant His-fusion proteins

Blanca de las Rivas,[†] José Antonio Curiel,[†] José Miguel Mancheño,[‡] and Rosario Muñoz*[†]

Departamento de Microbiología, Instituto de Fermentaciones Industriales[†] and Grupo de Cristalografía Molecular y Biología Estructural, Instituto de Química-Física "Rocasolano" [‡], CSIC, 28006- Madrid, Spain

*To whom correspondence should be addressed. Phone: +34-91-5622900. Fax: +34-91-5644853. E-mail: rmunoz@ifi.csic.es

Abstract

In this work we have constructed two novel expression vectors, designated as pURI2 and pURI3, which enable parallel cloning of a given target gene for producing recombinant His-fusion proteins. The vectors were created using the well-known pT7-7 and pIN-III-A3 plasmids as their template. The same DNA fragment containing the His-tag, enterokinase cleavage site, and a *Not*I unique site, as well as keeping the *Hin*dIII unique restriction site, was introduced in both vectors. These vectors have been designed to avoid the enzyme restriction and ligation steps during the cloning. The unique *Not*I site was introduced to facilitate the selection of the adequate recombinant plasmid. Parallel cloning of the same PCR fragment can be carried out since both vectors shared the same leader sequence. The described strategy avoids tedious cloning efforts into different expression vectors and represent a highly efficient means of cloning. To validate our vectors, we have cloned one target gene in both vectors and used expression and purification techniques to obtain the recombinant target protein. We herein show that both vectors function effectively in all the required experimental steps–cloning, expression, purification and cleavage.

Introduction

The genomic and structural genomic communities have driven the development of high-throughput cloning, expression and purification technologies to a large extent. The engineering of plasmids and expression vectors is one of the major tasks in a molecular biology laboratory. Many sophisticated plasmid constructions cannot be easily and rapidly engineered in a routine way. For example, the necessary endonuclease restriction sites might not be available or could be unsuitable for the desired constructions. Thus, a restriction enzyme-free cloning strategy is highly desirable. Several attempts to develop DNA cloning methods without the use of restriction enzymes and independent of DNA ligation have already been described (*1*, *2*) and have been made commercially available. Some protocols incorporate the ligation-independent cloning (LIC) of target genes. LIC possesses several advantages; it eliminates the use of restriction endonuclease digestion and ligation of PCR products, allowing any gene to be cloned into the vector regardless of its sequence. In LIC, PCR primers are designed to append sequences that, after treatment with T4 DNA polymerase in the presence of a single deoxyribonucleotide triphosphate, generate 12 to 15-base pair overhangs that are complementary to overhangs generated in the vector. These overhangs anneal sufficiently strongly to allow the transformation of hosts without ligation of the fragments; host repair enzymes ligate the introduced plasmid. In addition, another LIC method allows the insertion of PCR fragments in between any two nucleotides within a target plasmid. The only requirement is that the amplified fragment must be embedded between DNA sequences homologous to the site in which the integration is planned (*3*).

The purification of expressed proteins is accelerated by attachment of affinity tags to the N- or C-terminus of expressed proteins. They can provide hundred or even thousand-fold purification from crude extracts without prior steps to remove nucleic acid or other cellular material. The mild elution conditions employed make affinity tags useful for purifying individual proteins and specially protein complexes. There are several commercially available affinity tags, such as the peptide hexahistidine, glutathione *S*-transferase, maltose-binding protein, covalent yet dissociable, FLAT, etc. among others (*4*). Tagging with hexahistidine residues has several merits: low levels of toxicity and immunogenicity, small size, and no electric charge (*5*). Usually, His-tag provides good yields of tagged protein from inexpensive, and high capacity nickel-chelating resins (*4, 6*).

As these affinity tags may influence the tertiary structure of recombinant proteins and may interfere with protein function, removal of this tag seems advantageous and consequently a specific protease cleavage site need to be introduced in the vector. The enterokinase is a highly specific protease that cleaves a five-amino-acid recognition sequence. An enterokinase (EK) site allows removal of the affinity tag only, and does not leave any extra residues after cleavage on the target protein as compared to the native protein.

As many of the commercially-available vectors encoding affinity tags require different cloning and purification step for each vector, we have developed two *E. coli* expression vectors for enzyme restriction- and ligation-independent cloning that allow for parallel cloning from a single PCR product and thus, the recombinant protein can then be purified by using the same protocol.

Materials and Methods

Strains. *Escherichia coli* DH5αF´ [F´/*end A1 hsdR17* ($r_k^-m_k^+$) *supE44 thi-1 recA1 gyrA*(Nal^r) *relA1 Δ*(*lacIZYA-argF)U169 deoR* (*ϕ80dlacΔ(lacZ)M15;* Promega] was used for all DNA manipulations and for expression in pURI2 vector. *E. coli* JM109 (DE3) [*endA1 recA1 gyrA96 hsdR17 supE44 relA1 thi*Δ (*lac-pro*) F´ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15) λ*c*I857 *ind1 Sam/ nin5 lacUV5*-T7 gene 1; Promega] was used for expression in pURI3 vector. *Lactobacillus hilgardii* CECT 4786^T (ATCC 8290) was purchased from the Spanish Type Culture Collection (CECT). *Lactobacillus brevis* RM273 was isolated from wine (*7*). Plasmids pIN-III(lpp^p-5)A3 (*8, 9*) and pT7-7 (USB) are expression vectors that allow the hyperexpression of the desired protein upon induction with isopropyl-β-D-thiogalactopyranoside (IPTG).

L. brevis and *L. hilgardii* strains were routinely grown in MRS medium (*10*) at 30 ºC without shaking. *E. coli* strains were cultured in Luria-Bertani (LB) medium (*11*) at 37 ºC and 200 rpm. When required, ampicillin or chloramphenicol were added to the medium at a concentration of 100 or 34 μg ml^{-1}, respectively. Chromosomal DNA, plasmid purification, and transformation of *E. coli* were carried out as described elsewhere (*12*).

Construction of pURI2 and pURI3 vectors. The pUIR2 vector has been constructed starting from plasmid pIN-III(lpp^p-5)A3 (*8, 9*). First we performed a PCR using *L. brevis* RM273 DNA as template. We amplified a 230 bp from a non-coding intergenic DNA region (unpublished results). To perform the PCR we used two long synthetic primers, primers 246 and 245. The forward primer 246, from 5´to 3´ direction, contained:

22 nucleotides that paired the pIN-III(lpp^{p}-5)A3 sequence from the unique *Xba*I recognition site, a six poli-His tag, the enterokinase recognition site, and, 35 nucleotides pairing the *L. brevis* non-coding sequence (246, 5´-ACTCTAGAGGGTATTAATAATGGGGGGTTCT*CATCATCATCATCATCATCAT*GGT**GAC GATGACGATAA**GATGCCTGCTACTGCTAATCGCTATCATTTTGGCGG) (the *Xba*I recognition site is underlined, the enterokinase recognition site is written in bold, and the poli-His tag in italics).

The reverse primer 245 contains from 3´to 5´ direction, a *Hin*dIII restriction site, several stop codons arranged in tandem, a *Not*I restriction site, and 27 nucleotides pairing the *L. brevis* sequence (245, 5´-GGATCCAAGCTTAGTTAGCTATTATGCGTAGCGGCCGCAGATTCAGTAAAGCCT CGTGTCGCTCG) (the *Hin*dIII and *Not*I restriction sites are underlined). The purified PCR fragment was digested with *Xba*I and *Hin*dIII, whose target sequences were incorporated into the primers, and cloned into pIN-III(lpp^{p}-5)A3 digested with the same restriction enzymes. The resulting plasmid of 7785 bp, pURI2 (Figure 1), encodes expression of a leader sequence consisting of a N-terminal methionine followed by three spacer amino acids, a six-histidine affinity tag, a spacer glycine residue, and the five-amino acid enterokinase recognition site, DDDDK, followed by an initial protein methionine, under the control of the lpp^{p}-5 and lac^{PO} promoters, which can be induced at high levels by IPTG. The sequence and the site of insertion were verified by restriction analysis and DNA sequencing.

Expression vector pURI3 was constructed based on the commercial expression vector pT7-7 (USB) (*13*) following the same strategy described above. We used *L. brevis* DNA as DNA template in the PCR reaction with long primers 244 and 245. Primer 244 was

identical to primer 246, except for that the nucleotides based on pIN-III(lpp^{p}-5)A3 sequence were substituted by nucleotides based on the pT7-7 sequence containing a *Nde*I restriction site (244, 5´-AGATATACATATGGGGGGTTCT*CATCATCATCATCATCATCAT*GGT**GACGATGACGATAAG**ATGCCTGCTACTGCTAATCGCTATCATTTTGGCGG) (the *Nde*I restriction site is underlined, the enterokinase recognition site is written in bold, and the poli-His tag in italics). As reverse primer we used primer 245. The purified PCR fragment was digested with *Nde*I and *Hin*dIII, and cloned into pT7-7 digested with the same enzymes. The resulting plasmid of 2690 bp, pURI3 (Figure 2) contained the same expression leader sequence found in pURI2 under the control of the T7 RNA polymerase ϕ promoter, which can be induced at high levels by IPTG. The pURI3 sequence was verified by restriction analysis and DNA sequencing. The pURI3 expression plasmid was amplified in the *E. coli* DH5α strain and then transferred, for protein production, to the host JM109(DE3) (pLysS) *E. coli* strain. These cells contain λDE3 as a permanent lysogen and the T7 RNA polymerase under the control of the *lac*UV5 promoter inducible by IPTG.

Cloning, expression and purification of the target protein. To clone into the pURI2 and pURI3 expression vectors, the ornithine transcarbamoylase (OTC) gene sequence from *L. hilgardii* was amplified by *Pfu* DNA polymerase using the following oligonucleotides, forward 282 (5´-*CATCATGGTGACGATGACGATAAGatg*acaaaagattttagacaaaacg) and reverse 283 (5´-*AAGCTTAGTTAGCTATTATGCGTA*ctaggaatgaataggttacccaaag) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the OTC

gene sequence are written in lowercase letters). The amplification was for 20 cycles with the following conditions: 95 ºC 30 s, 55 ºC 1 min, 72 ºC for 3 min. After amplification, the PCR product was gel purified. This 1-kb purified PCR product was inserted into pURI2 and pUIR3 by using the restriction enzyme- and ligation-free cloning strategy described by Geiser et al. (2001) (*3*). Briefly, the purified PCR product was used as homologous primer pair in a PCR reaction using pURI2 or pUIR3 vectors as template. The product of this PCR was digested with *Dpn*I that exclusively restricts methylated DNA, and later, with *Not*I, an enzyme that only cuts the original copies of pURI2 or pURI3 vectors. *Dpn*I digestion was made directly in the PCR reaction buffer, and *Not*I digestion was made in buffer H (Roche). *E. coli* cells were transformed directly with the digestion product (Figure 3).

The pURI2 expression plasmid was amplified in the *E. coli* strain DH5α and pURI3 in the host JM109(DE3) (pLysS) *E. coli* strain. Cells carrying the recombinant plasmid were grown at 37 ºC in Luria-Bertani media containing ampicillin (100 μg/ml final concentration) and chloramphenicol (34 μg/ml final concentration) when required, and induced by adding IPTG (0.4 mM final concentration). After induction, the cells were grown at 22 ºC during 20 h and collected by centrifugation. Crude extracts were prepared by French Press lysis (three cycles at 1100 p.s.i.) of cell suspensions obtained by suspending the frozen cell paste with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl. The insoluble fraction of the lysate was removed by centrifugation at 47 800 *g* for 30 min at 4 ºC.

The cell homogenate, obtained by French Press lysis of the *E. coli* cells, was then applied to a His-Trap^{TM}-FF crude chelating affinity column (Amersham Biosciences, Uppsala, Sweden) using an ÄKTA-Prime system (Amersham Pharmacia Biotech). The column was equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 10 mM

imidazole, to improve the interaction specificity in the affinity chromatography step. The bound enzyme was eluted by applying a continuous gradient of imidazole concentration, from 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 10 mM imidazole to 20 mM Tris-HCl, pH 8.0, 100 mM NaCl containing 500 mM imidazole. Fractions containing the eluted OTC were pooled and the protein was then dialyzed against 20 mM Tris-HCl, pH 8.0, 100 mM NaCl. The concentration of OTC was estimated using the Bio-Rad protein assay. Protein at a concentration of 2.5 μg/ml was incubated with one unit of recombinant enterokinase (Novagen) and allowed to incubate at 21 ºC for 4 or 8 hours. The sample was then run on a SDS gel to check the cleavage.

OTC activity was determined by measuring the formation of citrulline from ornithine and carbamyl phosphate according to a method described previously (*14*).

Results and Discussion

Characterization of pURI2 and pURI3 expression vectors. The emerging field of structural genomics embraces many diverse objectives, but in general depends on the generation of protein structural information at fast rate. To achieve this objective, every step involved in protein production must be efficiently optimised, including the cloning and expression of the target gene, and further purification steps of the target protein. The importance of these aspects is revealed by the fact that there are numerous commercially- and non-commercially available *E. coli* expression vectors that incorporate different backbones, promoters, tag positions and cloning procedures. As the final yield of protein production is dependent on the vector used, we set out to create two novel expression

vectors with the same cloning procedure and identical initial purification setup. The vectors pIN-III(lpp^{p}-5)A3 and pT7-7 were used as the basis for the new expression vectors.

Expression vector pIN-III(lpp^{p}-5)A3 was chosen since it contains the promoter of the gene for the major outer membrane lipoprotein (*lpp*) that is considered to be one of the most efficient promoters in *E. coli* (*9*). In the high expression cloning pIN-III(lpp^{p}-5)A3 vector, a *lac* promoter-operator fragment was inserted downstream of the *lpp* promoter (Figure 1). Therefore, transcription of a cloned gene in pIN-III(lpp^{p}-5)A3 occurs in the presence of *lac* inducers since this is regulated not only by the strong *lpp* promoter but also by the *lac* promoter-operator.

Similarly, pT7-7 was selected since it contains the inducible bacteriophage T7 RNA polymerase promoter that provides the basis for a very active and selective gene expression system in *E. coli* (*15*). A convenient configuration has the gene for T7 RNA polymerase in the chromosome with the target gene in a multicopy plasmid under the control of a T7 promoter. However, this configuration can be used only if the target gene is not too toxic, because basal level of T7 RNA polymerase activity in the uninduced state provides significant transcription of the target gene. Bacteriophage T7 lysozyme is a specific inhibitor of T7 RNA polymerase, and it can be used to lower the basal activity of T7 RNA polymerase in the T7 gene expression systems. Low levels of T7 lysozyme supplied by plasmid pLysS, which is compatible with the vector for expressing genes from a T7 promoter, are sufficient to stabilize many target plasmids and yet allow high levels of target protein to be produced upon induction of T7 RNA polymerase (*16*).

To improve these traditional expression vectors, we included a N-terminal His-tag that allows convenient purification of the protein from crude cell extracts (*4*), and also an enterokinase cleavage site that leaves no extra residues after cleavage on the target protein

as compared to the native protein. In order to establish a cloning protocol for both modified vectors, we decided to make use of the strategy described by Geiser et al. (2001) (*3*), an adaptation of the QuickChange™ Site-Directed Mutagenesis protocol (Stratagene). This is a method that allows the insertion of PCR fragments in between any two nucleotides within a target plasmid. As the authors successfully integrated PCR fragments up to 1117 pb, we decided to included in the modified pIN-III(lpp^{p}-5)A3 and pT7-7 vectors a 230 bp sequence in order to facilitate the integration of larger DNA fragments. This 230 bp sequence was chosen from a *L. brevis* non-coding intergenic region (unpublished results), although any other non-coding piece of DNA could be also chosen. To construct the improved vectors we used long oligonucleotides pairing the *L. brevis* sequence and that contained all the required elements (poli-His tag, enterokinase recognition site, etc.) as described in Material and Methods section. Therefore, by using primers 246 and 245 we construct expression vector pURI2 (Figure 1) based on pIN-III(lpp^{p}-5)A3, and by using primers 244 and 245 we construct expression vector pURI3 (Figure 2) based on pT7-7 vector. Both expression vectors shared a common 310 bp fragment from the ATG start codon upstream the poli-His tag coding sequence to the *Hin*dIII restriction site (Figure 1 and 2). This common DNA fragment encodes for: a leader sequence consisting of a N-terminal methionine followed by three spacer amino acids, a six-histidine affinity tag, a spacer glycine residue, and the five-amino acid enterokinase recognition site, DDDDK, followed by an initial protein methionine, the 230 pb non-coding intergenic *L. brevis* sequence, a rare-cut *Not*I restriction site, and four stop codons arranged in tandem, and, finally a *Hin*dIII restriction site (Figure 1 and 2).

Cloning, expression, and purification of recombinant OTC protein. Figure 3 shows the general procedure for parallel cloning into pURI2 and pURI3 vectors. To clone into the pURI2 and pURI3 expression vectors, PCR products of the gene of interest need to be generated with specific overhangs that are complementary to the integration site sequence of the vectors. Since both vectors shared common sequence, the PCR oligonucleotides primers could be designed in order to be used with both expression vectors. As a general rule, to clone any target protein into both vectors the sequence of the forward primer should be 5´- CATCATGGTGACGATGACGATAAGATG (the last three nucleotides encode the first methionine residue of the target protein) followed by nucleotides pairing the sequence from the second amino acid residue of the target protein (Figure 3). Similarly, the reverse primer, also common for both expression vectors, could be the sequence 5´-AAGCTTAGTTAGCTATTAAGCGTA followed by nucleotides pairing the sequence up to the stop codon of the target protein (Figure 3). The nucleotide sequence of the gene encoding the target protein is amplify by PCR using these oligonucleotides and *Pfu* DNA polymerase. After the reaction, the fragment is added to the methylated recipient template plasmid. After the denaturation step, the homologous parts of the PCR fragment hybridise to the defined integration site. The fragments are elongated in vitro by the *Pfu* DNA polymerase. The elongated and modified strands are not methylated. This cycle is repeated 30 times. Through cycling, copies of the unmethylated DNA embedding the PCR fragment are produced. *Dpn*I is an enzyme that exclusively restricts methylated DNA. An additional screening tool is a *Not*I digestion of the template plasmid, only if a *Not*I restriction site is not included in the target gene sequence. However, this method is suitable for cloning any gene, even genes with internal *Not*I restriction sites. The *Dpn*I and *Not*I digested PCR product can be transformed directly into *E. coli* and recovered

(Figure 3). Because each individual step of this procedure can be done in parallel, including the PCR, *Dpn*I/*Not*I digestions, and the transformation, the protocol is suitable for parallel pURI2/pURI3 recombinant plasmid constructions.

The utilization of pURI2 and pURI3 expression vectors was assessed by expressing the ornithine transcarbamoylase (OTC) gene sequence from *L. hilgardii*. Primers 282 and 283 were designed to amplify the target OTC gene and append the appropriate sequences to the ends of the PCR products to allow its introduction into pURI2/pURI3 by the enzyme-restriction and ligation-independent cloning strategy explained above. Each of the constructs was transformed into *E. coli* DH5α. The cloning efficiency of each vector, defined as the percentage of colonies that possessed the target protein, was evaluated. Efficiencies of 75 and 90% were obtained for pURI2 and pURI3 vectors, respectively. It should be mentioned, that long time digestion with *Dpn*I (4 h) and *Not*I (2 h) were required to reduce sufficiently the background of colonies bearing pURI2 or pURI3 vectors. Success lies mainly in the degree of degradation, and secondarily in the linearization of the original vector, and therefore, the *Not*I restriction site was included in the designed expression vector. In addition, cloning efficiencies decreased according to the increase in the size of the gene encoding DNA fragment. Gene sequences up to 3 kb were successfully introduced into pURI2/pURI3 vectors (data not shown).

E. coli DH5α and JM109 (DE3) strains were used for the cloning and expression of genes in pURI2 and pURI3 vectors. Expression vector pURI2 can be used in a single strain of *E. coli* in all steps from plasmid construction to the expression of the target gene. To express the target gene in the recombinant pURI3 plasmids, although it was initially cloned into *E. coli* DH5α strain, it need to be transformed into DE3 cells. In these cells, the T7 polymerase gene is under the control of the IPTG-inducible lacUV5 promoter. Therefore,

the recombinant plasmid pURI3-OTC was transformed in *E. coli* JM109(DE3) (pLysS). OTC protein was expressed by IPTG induction, and following expression, the cultures were lysed, and centrifuged to sediment insoluble cell debris. As expected for vector based on T7 polymerase, pURI3 generate very strong overexpression of cloned genes (*15, 17*) (Figure 4B). Therefore, a significant OTC synthesis was observed in pURI3-OTC cell cultures after induction with 1 mM IPTG (Figure 4B). Otherwise, pURI2-OTC cell extract did not showed an apparent protein of the expected size, 38.3 kDa (Figure 4A). Even not obvious protein hyperproduction was achieved, the supernatant of sonicated cell lysates prepared from *E. coli* DH5α harbouring the recombinant plasmid pURI2-OTC showed OTC activity, whereas extracts prepared from control cells containing the vector plasmid alone did not (data not shown). Optimisation of soluble yields was not performed in this study, as this can vary greatly from protein to protein.

Purification trials demonstrated that the His-tag and the EK cleavage site function properly. The functionality of the His-tag and the EK cleavage site of pURI2 and pURI3 vectors was demonstrated by purifying OTC protein. OTC was purified on a His-Trap™-FF crude chelating affinity column and eluted with a continuous gradient of imidazole. Highly purified OTC protein was obtained from both expression vectors (Figure 4). In pURI2-OTC (Figure 4A) in spite of that soluble OTC protein showed a very faint band in the soluble fraction, it could be purified at detectable quantity (Figure 4A, line 3).

Generally, affinity tags are removed after purification as they can interfere with either the protein function or with downstream processes. Each construct above described has an EK cleavage site which can be used to remove the affinity tag, leaving the native protein. To validate the EK cleavage site, the purified OTC protein was dialyzed and

digested with EK. As showed in Figure 5 commercial EK (Novagen) cleaved the target protein efficiently, as half of the purified OTC was cleaved in 8 h at 21 ºC.

Conclusion

In summary, the results presented herein showed that the developed pURI2 and pURI3 expression vectors allow the overexpression and further purification of native proteins. Because of the variability of biological samples, no one vector is ideal for all proteins. In this sense, the expression vectors developed here present the advantage that the same PCR product can be introduced into both expression vectors following the same strategy. Additionally, this strategy allows the introduction of a 1 kb DNA fragment having at each side only short regions of homology covering the insertion point. We also have successfully introduced PCR fragments up to 3 kb into pURI2/pURI3 vectors by using this strategy. A N-terminal His-tag allows convenient purification of the native protein directly from crude cell extracts. The His-tag can be cleaved off from the purified protein by utilizing an enterokinase cleavage site that yield the native protein. The described strategy allows parallel cloning of any gene, avoiding tedious cloning efforts into different expression vectors.

Acknowledgment

This work was supported by grants AGL2005-00470 (CICYT) and S-0505/AGR/000153 (CAM). The technical assistance of M.V. Santamaría is greatly appreciated. J.A Curiel is a recipient of a predoctoral fellowships from the MEC. The vectors pURI2 and pURI3 are available from the authors upon request.

References and Notes

(1) Tillet, D.; Neilan, B. A. Enzyme free cloning: a rapid method to clone PCR products independent of vector restriction enzyme sites. *Nucl. Acids Res*. **1999**, 27(19), e26.

(2) Tseng, H. DNA cloning without restriction enzyme and ligase. *BioTechniques* **1999**, 27 (6), 1240-1244.

(3) Geiser, M.; Cèbe, R.; Drewello, D.; Schmitz, R. Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *BioTecniques* **2001**, 31(1), 88-92.

(4) Lichty, J. J.; Malecki, J. L.; Agnew, H. D.; Michelson-Horowitz, D. J.; Tan, S. Comparison of affinity tags for protein purification. *Protein Expr. Purif.* **2005**, 41(1), 98-105.

(5) Masuda, J.; Takayama, E.; Satoh, A.; Kojima-Aikawa, K.; Suzuki, K.; Matsumoto, I. A novel expression vector, designated as pHisJM, for producing recombinant His-fusion proteins. *Biotechnol. Lett*. **2004**, 26(20), 1543-1548.

(6) Hochuli, E. Purification of recombinant proteins with metal chelate adsorbent. In: Setlow, J. K. (Ed.), Genetic Engineering, Principle and Methods. Plenum Press, New York, **1990**, Vol. 12, pp. 87-98.

(7) Moreno-Arribas, M. V.; Polo, M. C.; Jorganes, F.; Muñoz, R. Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. *Int. J. Food Microbiol.* **2003**, 84(1), 117-123.

(8) Masui, Y.; Mizuno, T.; Inouye, M. Novel high level expression cloning vehicles: 10^4-fold amplification of *Escherichia coli* minor protein. *Bio/Technology* **1984**, 2, 81-85.

(9) Inouye, S.; Inouye, M. Up-promoter mutations in the *lpp* gene of *Escherichia coli*. *Nucl. Acids Res.* **1985**, 13(9), 3101-3109.

(10) De Man, J. C.; Rogosa, M.; Sharpe, M. E. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol*. **1960**, 23, 130-135.

(11) Sambrook, J.; Fritsch, E. F.; Maniatis, T. Molecular Cloning: a Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. **1989**.

(12) Muñoz, R.; López, R.; de Frutos, M.; García, E. First molecular characterization of a uridine diphosphate galacturonate 4-epimerase: an enzyme required for capsular biosynthesis in *Streptococcus pneumoniae*. *Mol. Microbiol*. **1999**, 31(2), 703-713.

(13) Tabor, S; Richardson, C. C. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA.* **1985**, 82(4), 1074-1078.

(14) Arena, M. E.; Manca de Nadra, M. C.; Muñoz, R. The arginine deiminase pathway in the wine lactic acid bacterium *Lactobacillus hilgardii* X_1B: structural and functional study of the *arcABC* genes. *Gene* **2002**, 301(1-2), 61-66.

(15) Studier, F. W.; Moffatt, B. A. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol*. **1986**, 189 (1), 113-130.

(16) Studier, F. W. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.* **1991**, 219(1), 37-44.

(17) Studier, F. W.; Rosenberg, A. H.; Dunn, J.J.; Dubendorff, J. W. Use of T7 RNA polymerase to direct the expression of cloned genes. Methods Enzymol. **1990**, 185, 60-89.

Figure captions

Figure 1. (A) Schematic representation of the expression vector pURI2 (7785 bp). (B) Sequence of pURI2 containing the leader sequence encoding region. The expression leader sequence consists of a N-terminal methionine residue followed by a three-amino acid spacer, a six-histidine affinity tag, a spacer glycine residue, and the five-amino acid enterokinase recognition site, DDDDK, followed by an initial protein methionine. The expression leader sequence is under the control of the lpp^{P}-5 and lac^{PO} promoters. Cleavage of expressed proteins by EK occurs between the lysine and methionine residues (KM). Restriction sites are indicated and underlined in the nucleotide sequence. Tandem stop codons are indicated by asterisks.

Figure 2. (A) Schematic representation of the expression vector pURI3 (2690 bp). (B) Sequence of pURI3 containing the leader sequence encoding region. The expression leader sequence consists of a N-terminal methionine residue followed by three-amino acid spacer, a six-histidine affinity tag, a spacer glycine residue, and the five-amino acid enterokinase recognition site, DDDDK, followed by an initial protein methionine. The expression leader sequence is under the control of the T7 polymerase ϕ promoter. Cleavage of expressed proteins by EK occurs between the lysine and methionine residues (KM). Restriction sites are indicated and underlined in the nucleotide sequence. Tandem stop codons are indicated by asterisks.

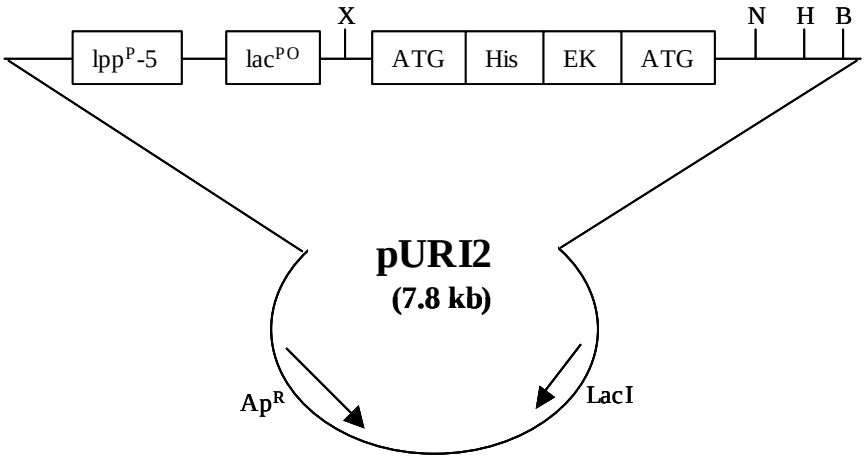
Figure 3. General procedure for ligation-independent cloning into pURI2 and pURI3 expression vectors. The primer sequences homologous to pURI2/pURI3 vectors are indicated. The *Hin*dIII restriction site on the reverse primer is underlined. The stop codons are indicated by asterisk.

Figure 4. Expression and purification of the 38.3 kDa OTC protein from *L. hilgardii* cloned into pURI2 and pURI3 vectors. SDS-PAGE analysis of soluble cells extracts of IPTG-induced cultures. (A) Expression on pURI2. Lane 1, *E. coli* DH5α (pURI2); lane 2, *E. coli* DH5α (pURI2-OTC); lane 3, *L. hilgardii* OTC from *E. coli* DH5α (pURI2-OTC) eluated after His-Trap^{TM}-FF crude chelating affinity column. (B) Expression on pURI3. Lane 1, *E. coli* JM109 (DE3) (pLysS) (pURI3); lane 2, *E. coli* JM109 (DE3) (pLysS) (pURI3-OTC); lane 3, *L. hilgardii* OTC from *E. coli* JM109 (DE3) (pURI3-OTC) eluated after His-Trap^{TM}-FF crude chelating affinity column. The 12% (A) and 10% (B) polyacrylamide gels were stained with Coomassie blue. The positions of molecular mass markers (Bio-Rad) are indicated on the left.

Figure 5. Cleavage of *L. hilgardii* OTC protein by enterokinase. OTC was purified on His-Trap^{TM}-FF crude chelating affinity column, eluted with buffer containing 125 mM imidazole, dialyzed overnight into 20 mM Tris-HCl, pH 8.0 buffer free of imidazole, and treated with enterokinase at 21 ºC. Lane 1, untreated; lane 2, 4 h incubation; lane 3, 8 h incubation. Broad-range molecular weight markers (Bio-Rad) are run on the left, and some positions are indicated.

Figure 1

A



B

*Ssp*I *lpp promoter*

AATA**TTGACA**ACATAAAAAACTTTGTGT**TATACT**TGTAACGCTACATGGAGATTAACTCAATCTAGCTAG

-35 -10

lac **promoter-operator**

AGAGGC**TTTACA**CTTTATGCTTCCGGCTCG**TATAAT**GTGTGGAATTGTGAGCGGATAACAATTTCACACA

-35 -10

*Xba*I **RBS**

GGAAACAGCTATGACCATGATTACGGATTCACTGGAACTCT**AGAGGG**TATTAATAATGGGGGGTTCT*CAT*

M G G S H

His tag **EK recognition site**

*CATCATCATCATCAT*GGTGACGATGACGATAAGATGCCTGCTACTGCTAATCGCTATCATTTTGGCGGGA

H H H H H G D D D D K M

CACCTGGCATCAGCCCCGCTAACCCTGCGGCCCAAAATCGGGTATACTGAACCTAACTCTTAGTAGAAAG

TAGGTCAGCGTCATTGGCAAATTACATCAAGGAAATTCGCGAACTCGTCGGTCACAAACCCATCATTCTT

*Not*I

AACGCTTCTGGTGGTCTGGTGACCAACGAGCGACACGAGGCTTTACTGAATCTGCGGCCGCTACGCATAA

*

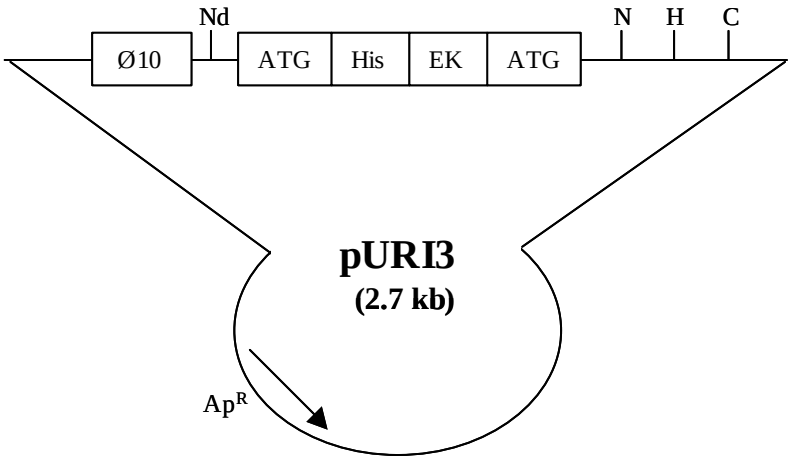
*Hin*dIII *Bam*HI

TAGCTAACTAAGCTTGGATCCGGCTGAGCAACGACGTGAACGCAA

* * *

Figure 2

A



B

T7 promoter

GATAGACTTCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGT

RBS *Nde*I **His Tag** **EK recognition**

TTAACTTTAAG**AAGGAG**ATATACATATGGGGGGTTCT*CATCATCATCATCATCATCAT*GGTGACGATGACGAT

M G G S H H H H H H G D D D D

site

AAGATGCCTGCTACTGCTAATCGCTATCATTTTGGCGGGACACCTGGCATCAGCCCCGCTAACCCTGCGG

K M

CCCAAAATCGGGTATACTGAACCTAACTCTTAGTAGAAAGTAGGTCAGCGTCATTGGCAAATTACATCAA

GGAAATTCGCGAACTCGTCGGTCACAAACCCATCATTCTTAACGCTTCTGGTGGTCTGGTGACCAACGAG

*Not*I *Hin*dIII *Cla*I

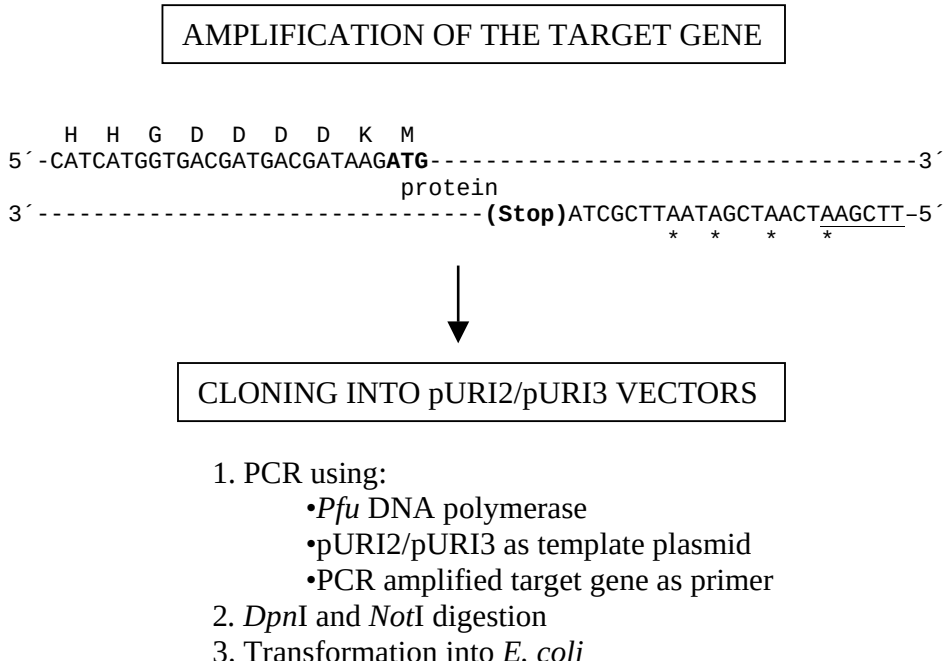
CGACACGAGGCTTTACTGAATCTGCGGCCGCTACGCATAATAGCTAACTAAGCTTATCGATGATAAGCTG

* * * *

TCAAACATGAGAATT

1 Figure 3

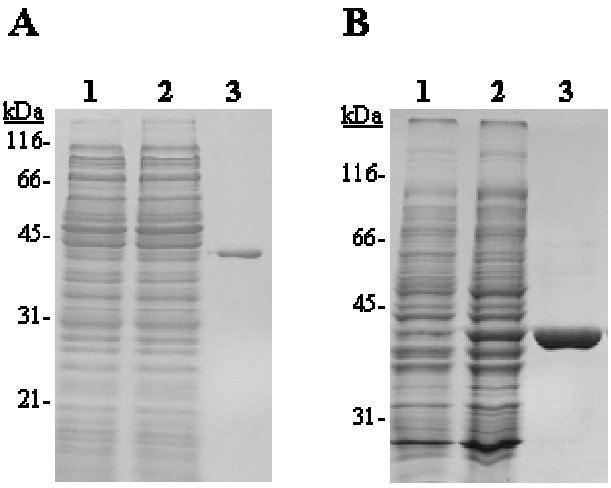
2


AMPLIFICATION OF THE TARGET GENE
H H G D D D D K M
5´-CATCATGGTGACGATGACGATAAGATG----------3´
protein
3´----------(Stop)ATCGCTTAATAGCTAACTAAGCTT-5´
CLONING INTO pURI2/pURI3 VECTORS
1. PCR using:
•Pfu DNA polymerase
•pURI2/pURI3 as template plasmid
•PCR amplified target gene as primer
2. DpnI and NotI digestion
3. Transformation into E. coli

1 Figure 4

2

3



1 Figure 5

2

3